Volume 230, number 1,2, 61–66 FEB 05687 March 1988

Characterization of the cDNA synthesized by avian retrovirus reverse transcriptase using 35 S avian myeloblastosis virus RNA and an exogenous bovine primer tRNA

Leila Sarih, Alejandro Araya and Simon Litvak

Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 rue Camille Saint Saens, 33077 Bordeaux cedex, France

Received 19 January 1988

Bovine $tRNA^{Trp}$ can be partially hybridized to the avian myeloblastosis virus (AMV) 35 S RNA at 37°C, in the presence of AMV RNA-dependent DNA polymerase (reverse transcriptase). This template-primer complex is active in the synthesis of viral cDNA. The size of the cDNA products synthesized in the in vitro reconstituted AMV system was determined by urea-polyacrylamide gel electrophoresis using a tRNA labelled at the 3'-end by yeast tRNA nucleotidyl transferase. The synthesized cDNA has a size of about 100 nucleotides and was shown by Southern blotting to be complementary to a specific sequence of the 5'-end of the retroviral genome. These results indicate that reverse transcriptase is able to anneal the exogenous primer tRNA at the 'primer-binding site' near the 5'-end of the long terminal repeat (LTR) of AMV RNA.

Reverse transcriptase; Avian myeloblastosis virus; $tRNA^{Trp}$; cDNA synthesis

1. INTRODUCTION

The synthesis of an intermediate double-stranded DNA is an early step in the replication cycle of retroviruses. This intermediary is synthesized by the viral RNA-dependent DNA polymerase (reverse transcriptase) which uses the RNA genome of the virus as template [1]. The first DNA strand to be synthesized is the complementary (−) strand of the viral genome. Reverse transcriptase starts cDNA synthesis by using a specific tRNA molecule as primer, $tRNA^{Trp}$ in the case of AMV [2]. Primer $tRNA^{Trp}$ is found in AMV viral particles hybridized to the viral genome at a distance of about 100 nucleotides from the 5'-end. The viral DNA polymerase initiates cDNA at the 3'-OH end of tRNA and proceeds to the 5'-end of the viral RNA generating a homogenous class of short complementary DNA (−) molecules called 'strong-stop cDNA'. Elongation continues via a 'jump' which is facilitated by the terminal redundancy found in the 5'- and 3'-ends of the retrovirus genome [1] (fig.1). The 5'-end of AMV RNA includes non-coding sequences: 'R', which is a redundant sequence also found at the 3'-end and 'U5', a characteristic sequence of the 5'-end. The 3'-end of the viral RNA includes the specific sequence 'U3' [3–5]. Strong-stop cDNA molecules of about 100 nucleotides in length have been obtained in vitro in various viral systems [6–8]. However, in all these experiments, the authors used either the 70 S viral RNA, or a heat-renatured (35 S RNA plus $tRNA^{Trp}$) primer-template complex.

The fact that primer tRNA annealing to the viral genome takes place over about 18 nucleotides from its 3'-end implies that the compact structure of tRNA has to be opened: hence, the factor(s) involved in the selection of the specific primer tRNA and its pairing to the retroviral genome remain(s) to be defined. The high affinity of AMV DNA polymerase for $tRNA^{Trp}$ [9] and the selection of the latter by reverse transcriptase from a mixture of chick embryo fibroblast tRNAs, to form a

Correspondence address: S. Litvak, Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 rue Camille Saint Saens, 33077 Bordeaux Cedex, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

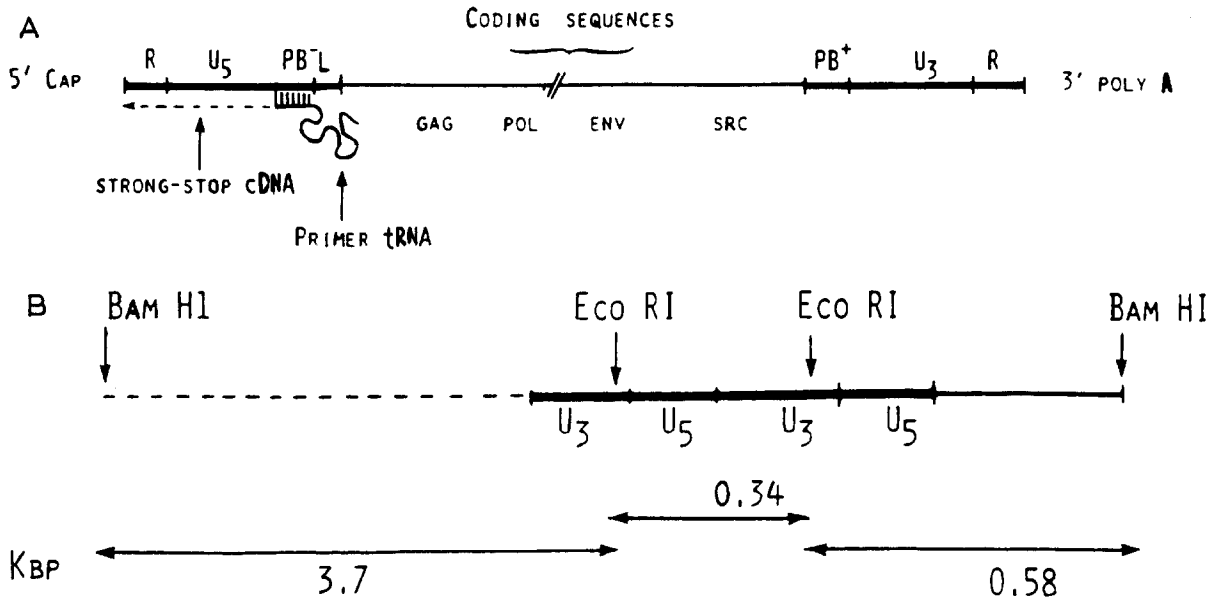

Fig.1. Schematic representation of the AMV genome (A) and LTR pRC11 plasmid after linearisation at the unique *Bam*HI restriction site (B). (A) R, redundant sequence; PB^- and PB^+, primer-binding sites for the synthesis of the cDNA (−) and (+) strands; L, sequence leader.

stable complex, shown by various techniques [10–13] led us to suggest that the viral enzyme was a very good candidate to play an important role in this process (review [14]).

In [15], we showed that beef liver $tRNA^{Trp}$ can be hybridized to the AMV 35 S RNA at 37°C in the presence of reverse transcriptase; this template-primer complex is active in the synthesis of DNA. Here, characterization of the viral cDNA synthesized in our reconstituted system was carried out, on the one hand, by urea-polyacrylamide gel electrophoresis using two procedures, (i) direct labelling of cDNA molecules with ^{32}P-labelled deoxynucleotides and (ii) employing $tRNA^{Trp}$ labelled at the 3′-end using the tRNA nucleotidyl transferase and, on the other, by using the radioactive 100-nucleotide-long cDNA, corresponding to the reverse transcription product synthesized by the reconstituted system, as a hybridization probe for Southern transfers of DNA fragments containing the U3 and U5 sequences.

2. MATERIALS AND METHODS

2.1. *Materials*

AMV and reverse transcriptase were obtained from Life Sciences (FL, USA). The enzyme was also purchased from Genofit. Viral 35 S RNA was isolated and purified by phenol extraction and separated from low-M_r RNAs as in [16]. Bovine $tRNA^{Trp}$ was purified by BD-cellulose chromatography [17]. Radioisotopes were purchased from Amersham. Restriction endonucleases were from Genofit and BRL. All other reagents were of the highest purity available.

2.2. *Methods*

2.2.1. Elongation of bovine $tRNA^{Trp}$ in the presence of AMV 35 S RNA by reverse transcriptase

Primer tRNA was labelled at the 3′-end in the presence of [α-^{32}P]ATP according to Silberklang et al. [18] using yeast tRNA nucleotidyl transferase (a kind gift from Dr R. Giégé, Strasbourg). Labelled tRNA was separated from degradation fragments and radioactive ATP by urea-polyacrylamide electrophoresis as described below. The tRNA was eluted from the gel in a buffer containing 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS and precipitated with ethanol.

Primer tRNA labelled at the 3′-end is used for the synthesis of viral cDNA in the following reaction mixture: 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 95 mM NaCl, 10 mM dithiothreitol, 100 μg/ml 35 S AMV RNA, 0.1 mM of each dNTP and 100 U/ml of AMV reverse transcriptase. The incubation was carried out at 41°C for 10 min and the reaction stopped by addition of a mixture of 1% SDS and 10 mM EDTA, followed by two phenolic extractions. After chromatography on Sephadex G-75 labelled cDNA was precipitated in 2.5 vols ethanol and stored at −20°C. This cDNA was redissolved in 6 μl of 10 mM Tris-HCl (pH 8), 1 mM EDTA and 3 μl of a solution containing 0.1% bromophenol blue, 0.1% xylene cyanol, 20 mM EDTA and 90% formamide,

heated for 2 min at 80°C and layered onto a denaturing 8% polyacrylamide gel containing 7 M urea, 1 mM EDTA and 100 mM Tris-borate (pH 8.3). After 1–2 h pre-electrophoresis, 3–4-μl samples were electrophoresed at 40 mA. The run was stopped when xylene cyanol had reached 3/4 of the way down the gel. The gel was fixed in 5% methanol/5% acetic acid solution, and dried under vacuum at 80°C for 2 h. Autoradiography was performed using an X-Omat AR-5 film (Kodak).

2.2.2. Agarose gel analysis of the restriction endonuclease-digested LTR pRC11 plasmid

Purification of pRC11 DNA was carried out as in [19]. 2 U restriction endonucleases were used to digest 2 μg DNA. In double-digestion experiments, the first enzyme (*Eco*RI) was inactivated by heating the sample at 65°C for 5 min, and the DNA was precipitated with ethanol and left at −80°C for 1 h. The DNA, recovered by centrifugation, was dried and dissolved in the reaction mixture of the second enzyme (*Bam*HI). The reaction was stopped by the addition of 0.1 vol. of 120 mM EDTA (pH 8.0), 50% glycerol and 0.25% bromophenol blue. DNA fragments were separated on a 1% agarose gel prepared in 40 mM Tris-acetate buffer (pH 8.0) plus 2 mM EDTA, and electrophoresis was carried out at 0.5 V/cm for 12–14 h. DNA bands were localized with ethidium bromide and the gel was photographed under UV light with a Polaroid MP-40 camera.

2.2.3. Southern hybridization analysis

Restriction endonuclease digestion products, separated by agarose gel electrophoresis, were transferred to nitrocellulose filters, hybridized and autoradiographed as described by Maniatis et al. [19] and Southern [20]. Annealing was performed for 16 h at 68°C in 6 × SSC, 10 mM EDTA, 5 × Denhardt solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. After drying the blot strip was exposed using X-Omat AR-5 film (Kodak).

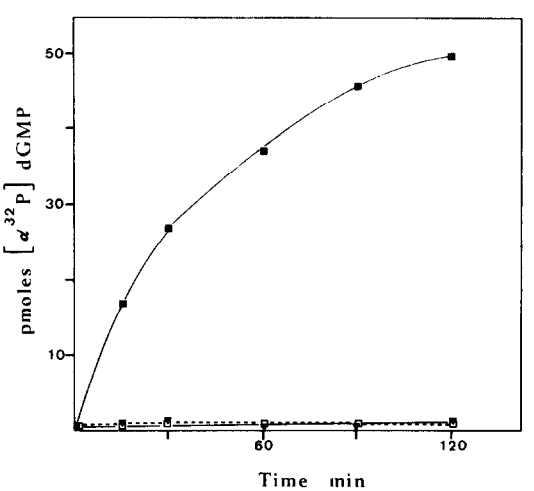

Fig.2. cDNA synthesis by AMV reverse transcriptase with primer tRNA-free 35 S AMV RNA in the presence and absence of exogenous tRNA. (■) Beef $tRNA^{Trp}$, (▼) yeast $tRNA^{Trp}$, (□) no tRNA added. Incubation was carried out at 37°C as described in the text.

3. RESULTS AND DISCUSSION

3.1. *Reverse transcriptase mediates cDNA synthesis with 35 S RNA as template in the presence of exogenous bovine tRNA primer*

The viral 35 S RNA deprived of its endogenous primer as described in section 2 does not serve as template for the AMV reverse transcriptase. Fig.2 shows that the retroviral DNA polymerase was able to synthesize DNA when exogenous bovine $tRNA^{Trp}$ was added to the incubation mixture at 37°C. No incorporation was observed in the absence of exogenous tRNA or the presence of yeast $tRNA^{Trp}$ (kindly donated by G. Keith, Strasbourg). The latter tRNA is recognized by AMV reverse transcriptase to form a stable complex (not shown) but cannot anneal to the viral RNA genome since its sequence at the 3′-end is very different from that of the bovine and avian $tRNA^{Trp}$ [21]. We have previously shown that this effect of reverse transcriptase is most probably mediated by the partial unwinding of the acceptor stem of tRNA, thus facilitating the partial pairing between template and primer [15]. However, a question remains to be answered: is the exogenous tRNA positioned at the primer-binding site on the retroviral genome? The following experiments were aimed at providing an answer to this question.

3.2. *Synthesis of strong-stop cDNA using 35 S AMV RNA as template*

Once the incubation conditions which allow the production of short cDNA strands with a purified 35 S AMV RNA as template had been determined, we incubated the viral template, largely devoid of endogenous primer as seen in fig.2, in the presence of exogenous bovine $tRNA^{Trp}$. This primer tRNA was labelled at the 3′-end with yeast tRNA nucleotidyl transferase. Hence, we could follow cDNA elongation of primer tRNA annealed to the genomic RNA in the presence of reverse transcriptase, by using unlabelled deoxynucleotide triphosphate precursors. As shown in fig.3, a radioactive band which barely enters the 8% acrylamide gel is observed after incubation with reverse transcriptase. Analysis of cDNA synthesized from a labelled tRNA primer under various conditions allowed us to determine its size. Fig.4B shows a cDNA band of 175 nucleotides which

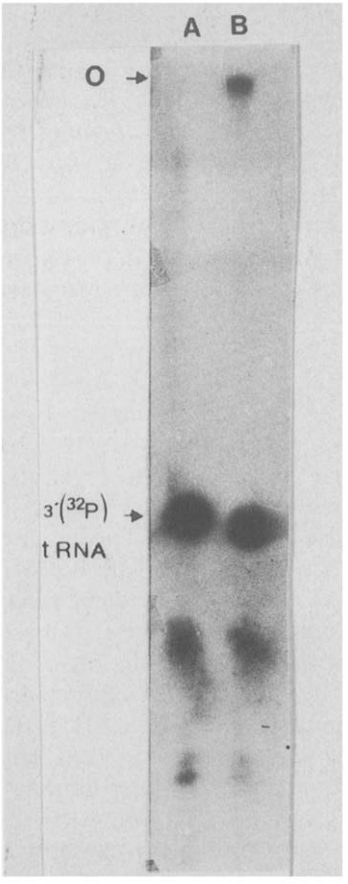

Fig.3. Elongation of beef $tRNA^{Trp}$, labelled at the terminal adenosine, by AMV reverse transcriptase. Primer bovine $tRNA^{Trp}$ was labelled by the tRNA nucleotidyl transferase, incubated with AMV reverse transcriptase and AMV 35 S RNA as described in section 2 and analyzed by polyacrylamide-urea gel electrophoresis. (A) 3'-end ^{32}P-labelled bovine $tRNA^{Trp}$ alone. (B) 3'-end ^{32}P-labelled bovine $tRNA^{Trp}$ elongated in the presence of AMV 35 S RNA and reverse transcriptase. Conditions for incubation and electrophoresis are detailed in the text.

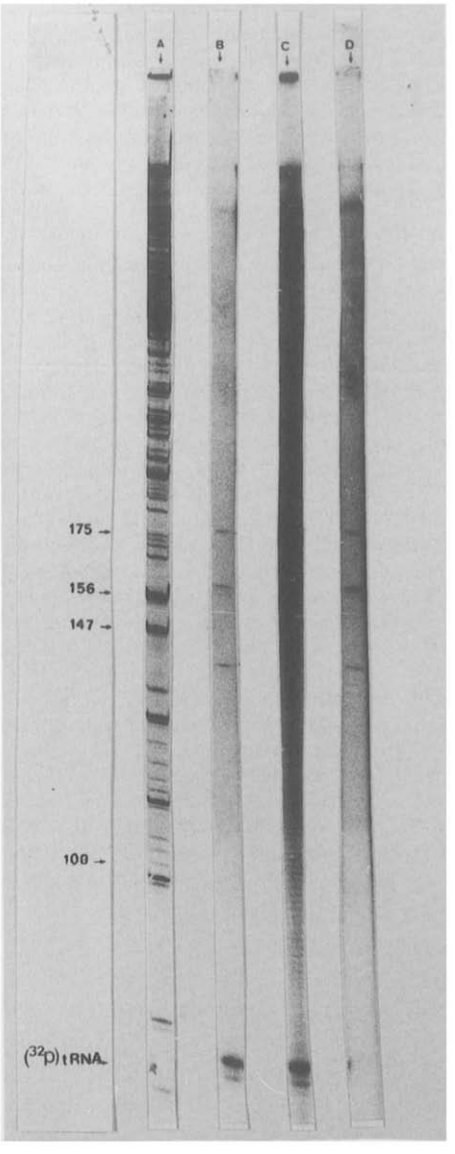

Fig.4. Analysis by urea-polyacrylamide gel electrophoresis of the cDNA synthesized under various conditions. (A) Molecular mass standards: M13 mp8 ssDNA was used as the template for DNA synthesis using ddCTP as chain terminator. (B) cDNA synthesized from the AMV 35 S RNA–3'-[^{32}P]$tRNA^{Trp}$ complex reconstituted in vitro in the presence of reverse transcriptase under the conditions described in the text. (C) cDNA synthesized from AMV 70 S RNA in the presence of [α-^{32}P]dNTP. (D) cDNA synthesized in the presence of [α-^{32}P]dNTP and AMV 35 S RNA with its endogenous primer.

coincides with the size of the strong-stop cDNA (primer tRNA plus newly synthesized DNA). Two other bands (156 and 140 nucleotides) could be abortive products or may originate from the reverse transcription of RNA molecules which have been degraded at their 5'-end. The same bands were observed whether we followed elongation with the 3'-labelled primer tRNA or in the presence of the viral 35 S RNA with its endogenous primer (fig.4D). It is interesting to point out that spontaneous chemical breakage of tRNA is observed under denaturing conditions as we have described previously [22]. These products may correspond to the high-M_r material observed in

fig.3B. AMV 70 S RNA was used as control (fig.4C). The observed background radioactivity could be due to heterogeneous synthesis resulting from the formation of RNA primers by the RNase H activity of reverse transcriptase [23].

3.3. *Hybridization of strong-stop cDNA synthesized with exogenous primer on nitrocellulose filters*

Viral radioactive cDNA was synthesized in the presence of bovine $tRNA^{Trp}$ and [α-^{32}P]dATP under the conditions described in section 2. The labelled product was assayed for hybridization with DNA fragments corresponding to the 5′- and 3′-ends of the proviral DNA: DNA fragments contained the specific U3 and U5 sequences of the AMV long terminal repeats (fig.1A). These fragments were obtained by *Eco*RI and *Bam*HI digestion of the LTR pRC11 plasmid (fig.1B) (the plasmid was a kind gift from Dr S. Saule, Lille).

The *Eco*RI 4.3 and 0.34 kbp fragments from pRC11 DNA, as well as the 0.58 kbp fragment obtained by double *Eco*RI-*Bam*HI digestion of the same plasmid, were submitted to 1% agarose gel electrophoresis. Fig.5A shows the plasmid pRC11 digestion products after incubation with *Eco*RI (lane 2) and a 580 bp fragment obtained from the same plasmid digested with *Eco*RI and *Bam*HI, electrophoresed and purified by electroelution before a second agarose electrophoresis run (lane 3). These fragments include the U5 region of the retroviral genome. The presence of the U5 sequence is confirmed as demonstrated when these fragments were transferred onto nitrocellulose filters and hybridized with a synthetic probe of 40 nucleotides corresponding to the U5 region (a kind gift from Dr P. Valenzuela, Chiron Co., San Francisco), which was labelled at the 5′-end with polynucleotide kinase. As depicted in fig.5B (lanes 1,2), the three above-mentioned digestion fragments showed hybridization with the radioactive probe.

The DNA fragments (3.7, 0.58, 0.34 kbp) obtained by double *Eco*RI-*Bam*HI digestion of the pRC11 plasmid were separated on a 1% agarose gel (fig.6, lane 2), transferred onto nitrocellulose filters and hybridized with the [^{32}P]cDNA synthesized in the reconstituted AMV system as described in section 2. Fig.6, lane 3 shows that the labelled viral cDNA synthesized in vitro hybridized to the 0.58 and 0.34 kbp DNA fragments which include the U5 sequence. No radioactive band was observed at the level of the 3.7 kbp fragment which includes only the U3 sequence of the 3′-end of the retroviral RNA. The 4.3 kbp band, which showed faint labelling, may correspond to a partial digestion product.

In summary, our results show that the viral cDNA, synthesized from the 35 S RNA-tRNA complex, reconstituted at 37°C by reverse

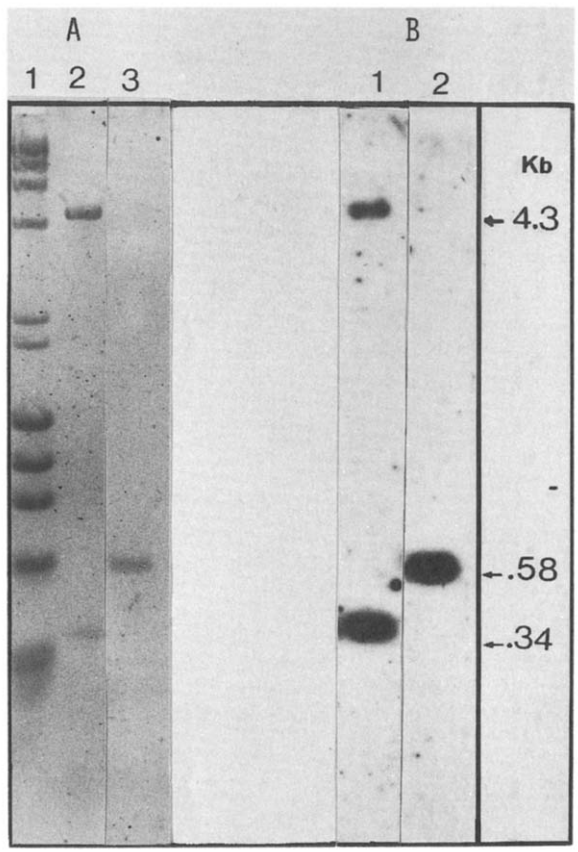

Fig.5. Analysis by agarose gel electrophoresis of restriction nuclease digested LTR pRC11 plasmid (A) and Southern blotting using a synthetic probe encoding part of the LTR U5 region (B). (A) Lanes: 1, DNA digested with *Hin*dIII and ϕX174 DNA digested by *Hae*III; 2, LTR pRC11 digested with *Eco*RI; 3, a 580 bp fragment obtained from the LTR pRC11 plasmid digested with *Eco*RI and *Bam*HI and electroeluted from a preparative gel (see scheme in fig.1B). (B) Lanes: 1, hybridization of the electrophoresed digestion products shown in lane 2 in (A) with a labelled 43-mer corresponding to the LTR U5 region; 2, hybridization of the 580 bp *Eco*RI-*Bam*HI fragment shown in lane 3 in (A) with the labelled 43-mer probe. Experimental conditions are described in the text or [19].

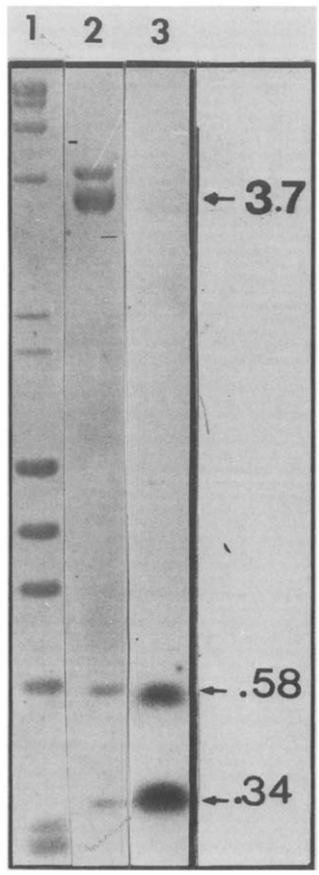

Fig.6. Hybridization of *Eco*RI- and *Bam*HI-digested LTR pRC11 plasmid with ^{32}P-labelled strong-stop AMV cDNA. Lanes: 1, DNA digested by *Hin*dIII and ϕX174 DNA digested by *Hae*III; 2, LTR pRC11 plasmid sequentially digested with *Eco*RI and *Bam*HI and electrophoresed in agarose gel as described in the text; 3, labelled strong-stop AMV cDNA synthesized, isolated and hybridized (Southern blotting) to the digested DNA shown in lane 2, as described in section 2.

transcriptase, has a size of about 100 nucleotides and comprises the complementary sequence of the 5′-region of the viral RNA. Thus, it can be concluded that AMV reverse transcriptase positions the exogenous bovine $tRNA^{Trp}$ on the primer-binding site of the retroviral genome.

Acknowledgements: This work was partially supported by the CNRS, NATO, Association pour la Recherche sur le Cancer (ARC) and University of Bordeaux II. The authors are grateful to Dr T. Heyman (Orsay) for helpful discussions and to Dr L. Tarrago-Litvak for helpful comments on the text. One of us (L.S.) was the recipient of a fellowship from the Fondation pour la Recherche Médicale.

REFERENCES

[1] Varmus, H.E. (1982) Science 216, 812–820.
[2] Faras, A.J., Taylor, J.M., Levinson, W.E., Goodman, H.M. and Bishop, J.M. (1971) J. Mol. Biol. 79, 163–183.
[3] Groner, B. and Hynes, N.E. (1982) Trends Biochem. Sci. 7, 400–403.
[4] Cullen, B.R., Raymond, K. and Ju, G. (1985) Mol. Cell. Biol. 5, 438–447.
[5] Weber, F. and Schaffner, W. (1985) EMBO J. 4, 949–956.
[6] Collet, M.S. and Faras, A.J. (1976) Proc. Natl. Acad. Sci. USA 73, 1329–1332.
[7] Friedrich, R., Kung, H.J., Baker, B., Varmus, H.E., Goodman, H.M. and Bishop, J.M. (1977) Virology 79, 198–215.
[8] Olsen, J.C. and Watson, K.F. (1982) Nucleic Acids Res. 10, 1009–1027.
[9] Panet, A., Haseltine, W.A., Baltimore, D., Peters, G., Harada, F. and Dahlberg, J.E. (1975) Proc. Natl. Acad. Sci. USA 72, 2535–2539.
[10] Hizi, A., Leis, J.P. and Joklik, W.K. (1977) J. Biol. Chem. 252, 6878–6884.
[11] Baroudy, B.M., Fournier, M., Labouesse, J., Papas, T.S. and Chirijkian, J.G. (1977) Proc. Natl. Acad. Sci. USA 74, 1889–1893.
[12] Panet, A., Weil, G. and Friis, R.R. (1978) J. Virol. 28, 434–441.
[13] Araya, A., Keith, G., Fournier, M., Gandar, J.C., Labouesse, J. and Litvak, S. (1980) Arch. Biochem. Biophys. 205, 437–448.
[14] Litvak, S. and Araya, A. (1982) Trends Biochem. Sci. 6, 361–364.
[15] Araya, A., Sarih, L. and Litvak, S. (1979) Nucleic Acids Res. 6, 3831–3843.
[16] Coffin, J.M. and Billeter, M.A. (1976) J. Mol. Biol. 100, 293–318.
[17] Fournier, M., Labouesse, J., Dirheimer, G., Fix, C. and Keith, G. (1978) Biochim. Biophys. Acta 521, 198–208.
[18] Silberklang, M., Gillium, A.M. and Rajhbandary, U.L. (1977) Nucleic Acids Res. 4, 4091–4108.
[19] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, NY.
[20] Southern, E. (1979) Methods Enzymol. 68, 152–171.
[21] Keith, G., Roy, A., Ebel, J.P. and Dirheimer, G. (1972) Biochimie 54, 1405–1426.
[22] Garret, M., Romby, P., Giegé, R. and Litvak, S. (1984) Nucleic Acids Res. 12, 2259–2271.
[23] Baltimore, D. and Smoler, D.F. (1972) J. Biol. Chem. 247, 7282–7287.